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STUDY OF *NOSEMA* SPP. IN THE TOMSK REGION, SIBERIA: CO-INFECTION IS WIDESPREAD IN HONEYBEE COLONIES

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Summary. Microsporidian protozoans *Nosema* are gut parasites that infect European honeybees (*Apis mellifera*) worldwide. In the Tomsk region, two species of microsporidia were registered in honeybees (*A. mellifera*): *N. apis* and *N. ceranae.* During the last 6 years, an increase in infections by *Nosema* in honeybees has been detected in the Tomsk region, while cases of mass bee colony deaths were rare (in 2016, two cases of winter losses of bee colonies in the northern districts of the Tomsk region have been reported by beekeepers). The infestation of bee colonies and apiaries have changed from 0% in 2012 to about 80% in 2016–2017. In 2013–2014, 60.0% of all infected apiaries were infected only with *N. apis*. In 2015–2017, most of the infected apiaries (52.2%) were infected with both species of microsporidia. Despite the predominance of co-infection in honeybees, replacement of *N. apis* by *N. ceranae* is not observed.

Key words: Microsporidia, Nosematidae, *Nosema apis*, *Nosema ceranae*, Hymenoptera, *Apis mellifera,* honeybee, Siberia, Russia.

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Резюме. Микроспоридии рода *Nosema* являются кишечными паразитами, которые поражают медоносную пчелу (*Apis mellifera*) во всем мире. В Томской области зарегистрировано два вида микроспоридий у медоносной пчелы: *N. apis* и *N. ceranae.* В течение последних 6 лет наблюдается увеличение зараженности медоносных пчел микроспоридиями рода *Nosema* на пасеках области, хотя случаи массовой гибели пчелиных семей отмечались редко (в 2016 году было зарегистрировано два случая гибели пчелиных семей после зимовки на пасеках двух северных районов Томской области). Зараженность пчелиных семей и пасек нозематозом изменялась от 0% в 2012 году до примерно 80% в 2016–2017 гг. Если в 2013–2014 гг. на 60% пасек от числа зараженных нозематозом был зарегистрирован только возбудитель *N. apis*, то в 2015–2017 гг. на большинстве зараженных пасек (52.2%) присутствовало оба возбудителя нозематоза. Несмотря на преобладание варианта заражения «ко-инвазия», замещения возбудителя *N. apis* видом *N. ceranae* не наблюдается.

INTRODUCTION

For honeybees, three species of microsporidia have been described: *Nosema apis*, *N. ceranae*, and *N. neumanii*. Microsporidia *N. apis* Zander, 1909, responsible for type A nosemosis, is a globally distributed parasite specific for the European honeybee, *Apis mellifera* L. (Zander, 1909). *N. apis* was for years considered to be the only disease agent of nosemosis in honeybee *A. mellifera* (Bailey, 1955; Natsopoulou *et al*., 2015). *N. ceranae* Fries et al., 1996, responsible for type C nosemosis, was first described in Asian honeybee, *Apis cerana* Fabricius, at the end of the twentieth century (Fries *et al*., 1996) and later in honeybees *A. mellifera* worldwide (Fries *et al*., 2006; Higes *et al*., 2006, 2009, 2010a; Huang *et al*., 2007; Klee *et al*., 2007; Martin-Hernandez *et al*., 2007; Chen *et al*., 2008, 2010; Williams *et al*., 2008; Invernizzi *et al*., 2009; Fries, 2010; Yoshiyama & Kimura, 2011; Medici *et al*., 2012; Ostroverkhova *et al*., 2016a, b). In 2017, new species of microsporidia, *N. neumanii* Chemurot et al., 2017, was identified in honeybees in Uganda (Chemurot *et al*., 2017). No information about distribution, prevalence, and clinical signs specific to the new microsporidia has been reported so far.

Nosema infection caused by *N. apis* is characterized mainly by dysentery, whereas *N. ceranae* is described to cause death of individuals and bee colonies not preceded by any visible symptoms (Martin-Hernandez *et al*., 2007; Higes *et al*., 2008, 2010b). *N. apis* infection is restricted to the midgut epithelium (Fries, 1988), whereas *N. ceranae* has also been detected in other bee tissues like malpighian tubules and hypopharyngeal glands by molecular methods (Chen *et al*., 2009). In addition, these species also differ morphologically, genetically, in their ability to adapt to temperature, survival, and effects on the host (Higes *et al*., 2007, 2010c; Martin-Hernandez *et al*., 2007, 2011; Cornman *et al*., 2009; Forsgren & Fries, 2010; Chen *et al*., 2013; Huang *et al*., 2013; Van der Zee *et al*., 2014; Pelin *et al*., 2015).

N. apis is an evolutionarily old parasite of the honeybee *A. mellifera*, and the host-parasite relationship is well balanced. The parasite is characterized by moderate virulence, and bee colonies can often cure themselves under favorable environmental conditions (Klee *et al*., 2007; Chen *et al*., 2009).

N. ceranae has only recently been described in a honeybee *A. mellifera* (Higes *et al*., 2006; Fries *et al*., 2006). The virulence of the parasite, as well as the role and effect of *N. ceranae* infection in bee colonies has been controversially discussed. Some authors have defined a moderate virulence of parasite *N. ceranae* similar as that of *N. apis*, for example in French and German bees (Forsgren & Fries, 2010; Fries, 2010; Gisder *et al*., 2010, 2017). In other reports, *N. ceranae* was characterized as a parasite with high virulence and may have caused large bee colony losses in the countries of the Mediterranean, e.g. in Spain and Greece (Higes *et al*., 2008, 2010b; Bacandritsos *et al*., 2010; Soroker *et al*., 2011). It is supposed that the climate may influence the virulence of the parasites, as well as the spread of microsporidia (Martín-Hernández *et al*., 2009; Genersch *et al*., 2010; Gisder *et al*., 2010, 2017; Dainat *et al*., 2012; Budge *et al*., 2015). However, the prevalence of *Nosema* in various climatic areas, as well as the long-temp dynamics of infection of honeybees by various species of *Nosema* are poorly understood. Obviously, more research is needed to elucidate the full effect of *N. ceranae* infection in *A. mellifera* colonies in different geographical areas and to understand if individual virulence levels and colony virulence levels differ between the two parasites.

Siberia characterized by a sharp continental climate is of interest to study how the spread of *Nosema* correlates with climatic conditions and how the disease moves to particularly remote northern areas. The objective of our research was to study the long-term dynamics of the infection of bee colonies with microsporidian *Nosema* in apiaries of different areas of the Tomsk region, Siberia in 2012–2017.

MATERIAL AND METHODS

We investigated the infestation of 316 bee colonies obtained from 207 apiaries of the Tomsk region, Siberia during 2012–2017 by *Nosema* infections. From fifty to seventy bees were randomly selected from each bee colony, and total sample (pooled worker samples) from the bee colony was examined for the presence of *Nosema* by molecular genetic methods.

DNA isolation and polymerase chain reaction (PCR) was carried out according to standard techniques (Hamiduzzaman *et al*., 2010). The primer sequences utilized to amplify the 218-bp fragment corresponding to the 16S ribosomal gene of *N. ceranae* were 218MITOC–FOR 5′–CGGCGACGATGTGATATGAAAATATTAA and 218MITOC–REV 5′–CCCGGTCATTCTCAAACAAAAAACCG. The primer sequences used to amplify the 321 bp fragment corresponding to the 16S ribosomal gene of *N. apis* were 321APIS–FOR 5′–GGGGGCATGTCTTTGACGTACTATGTA and 321APIS–REV 5′– GGGGGGCGT TTAAAATGTGAAACAACTATG (Martín-Hernández *et al.*, 2007; Hamiduzzaman *et al*., 2010). PCR products were analyzed on 1.5% (m/v) agarose gels and visualized using UV illumination (Gel Doc XR+, BioRad, Foster City, CA, USA). All analyses were carried out in duplicate with use positive and negative controls, and identical results were obtained.

RESULTS AND DISCUSSION

Infestation of bee colonies and apiaries with microsporidia *Nosema* in the Tomsk region in 2012–2017

The study of the long-term dynamics of infection of bee colonies and apiaries with microsporidian *Nosema* from 2012 to 2017 showed that the infection of both bee colonies and apiaries changed gradually from 0% in 2012 to about 80% in 2016–2017, when the highest degree of bee infestation was detected (Fig. 1). A rapid, uneven increase in the number of *Nosema* infected bee colonies and apiaries is observed over a relatively short 6-year period. In 2015, a sharp increase in infection of bee colonies and apiaries (more than 2 times) by *Nosema* was registered. If in 2014, the *Nosema* pathogen was detected in only about 20% of apiaries and bee colonies (of the total number studied), in 2015 *Nosema* was detected in more than 40% of apiaries and 60% of bee colonies. In the next period (2016–2017), there was a further increase in the extensiveness of the infection of honeybees by *Nosema* in apiaries in the Tomsk region, and the number of infected bee colonies and apiaries reached about 80% (Fig. 1).

The reasons for the rapid increase in the number of infected bee colonies and apiaries for the studied period could be different: weather conditions of different years, active import of bee colonies infested with *Nosema* spores, and the lack of treatment of bee colonies, etc. For example, absence of nosemosis in honeybees on apiaries in the Tomsk region in the first year of study is probably due to weather conditions in the summer of 2012. High summer temperatures and low humidity (abnormally hot weather) were noted in 2012; during the active growing season, 86 cases of exceeding the absolute maximum air temperature for a given day were recorded. Such extreme weather conditions, observed for the first time in the Tomsk region over a long period of observation, could affect the development of nosemosis in bees. This year there has been no case of infection of bee colonies by *Nosema*, and honey-bees were characterized by high flying activity and honey productivity.

In addition, there has been an increase in the total number of apiaries and bee colonies in recent years in the Tomsk region: over the past 15 years, the number of bee colonies has doub-led; beekeepers actively import bee colonies from different regions of Russia and neighboring

countries, in whose territory some dangerous bee diseases, including type C nosemosis, are common. For example, in 2013, *N. ceranae* was first registered in bee colonies that were imported to the Tomsk region from the southern regions of Russia already infected (Ostroverkhova *et al*., 2014).

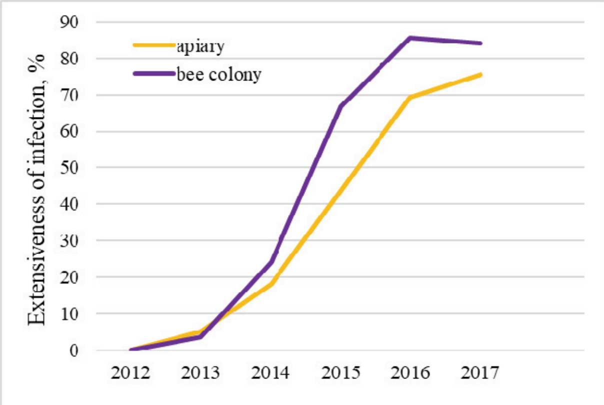


Fig. 1. Dynamics of infestation of bee colonies and apiaries with *Nosema* spp. in 2012–2017. Diagnosis of *Nosema* spp. was performed by PCR.

Thus, the results of our long-term study indicate a significant increase in the number of bee colonies infected with microsporidiosis in the Tomsk region in recent years. The obtained data are consistent with the results of studies conducted in different regions of Russia and some European countries (Bacandritsos *et al*., 2010; Genersch *et al*., 2010; Higes *et al*., 2010a; Stevanovic *et al*., 2011; Zinatullina *et al*., 2011, 2012; Martín-Hernández *et al*., 2012; Gisder *et al*., 2017). However, the causes of the spread of *Nosema* infection, especially *N. ceranae*, in honeybees are not fully understood. In this regard, it is of interest to study the species composition of *Nosema* parasites in apiaries of the Tomsk region, as well as the characteristics of interactions between microsporidia.

Spreading of *N. apis* and *N. ceranae* in apiaries of the Tomsk region

In order to determine which *Nosema* species was responsible for infections, each bee colony was analyzed by species specific PCR. In the Tomsk region, two species of microsporidia were registered in honeybees: *N. apis* and *N. ceranae* (Fig. 2).

In total, *Nosema* spp. DNA were detected in 195 out of 316 (61.7%) bee colonies. Of the *Nosema* positive bee colonies, 34.9% of the samples contained *N. apis* only, 23.1% of the samples *N. ceranae* only and 42.1% both *Nosema* species. Of the 207 apiaries analyzed, *Nosema* spp. were detected in honeybees from 79 apiaries (38.2%): 26.6% of infected apiaries were infected with *N. apis* only; 25.3% – *N. ceranae* only; in most apiaries (48.1%), both species of pathogen were detected.

In 2013–2015 (up to 2016), the spread of the pathogen *N. ceranae* (type C nosemosis) in apiaries in the Tomsk region was limited to only two southern districts (Tomsky and Shegarsky) (Fg. 3). However, in spring 2016, bee colonies infected with *N. ceranae* were identified in apiaries in two northern districts of the Tomsk region – Molchanovsky and Teguldetsky. In 2017, *N. ceranae* was detected in honeybees in apiaries of all southern districts and most northern areas (Fig. 3).

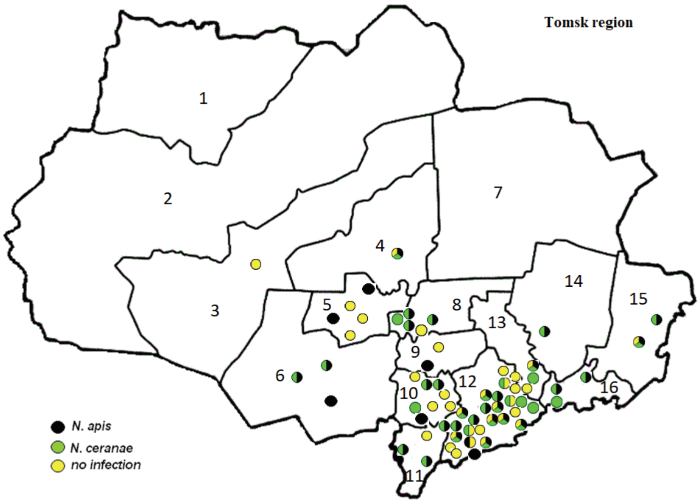
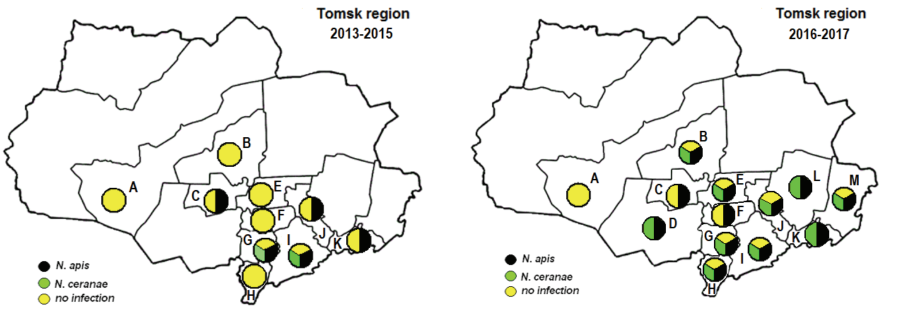


Fig. 2. Distribution of *Nosema* species in bee colonies (*Apis mellifera*) throughout the Tomsk region (dots A–I). Bee colonies not infected by *Nosema* are indicated in yellow; bee colonies corresponding to infection by *N. apis* or *N. ceranae* are indicated in black and green, respectively. Sectors in circles indicate representation cases (existence/absence) of an infection without frequency. Numbers (1–16) indicate the districts of the Tomsk region: northern districts – 1, Alexandrovsky; 2, Kargasoksky; 3, Parabelsky; 4, Kolpashevsky; 5, Chainsky; 6, Bakcharsky; 7, Verchneketsky; 8, Molchanovsky; 9, Krivosheinsky; 15, Teguldetsky; southern districts – 10, Shegarsky; 11, Kozhevnikovsky; 12, Tomsky; 13, Asinovsky; 14, Pervomaisky; 16, Zyryansky. Apiaries located at a distance of less than 20 km from each other are marked with one dot on the map. In the northern districts 1, 2, and 7, beekeeping is absent because of the harsh climatic conditions.



A B

Fig. 3. Distribution of *Nosema* in bee colonies (*Apis mellifera*) throughout the Tomsk region in 2013–2015 (A) and 2016–2017 (B). Bee colonies not infected by *Nosema* indicated in yellow; bee colonies corresponding to infection by *N. apis* or *N. ceranae* indicated in black and green, respectively. Sectors in circles indicate representation cases (existence/absence) of an infection without frequency. Literas (A–M) indicate the districts of the Tomsk region: A – Parabelsky; B – Kolpashevsky; C – Chainsky; D – Bakcharsky; E – Molchanovsky; F – Krivosheinsky; G – Shegarsky; H – Kozhevnikovsky; I – Tomsky; J – Asinovsky; K – Zyryansky; L – Pervomaisky; M – Teguldetsky.

It should be noted that in 2016, the winter losses of bee colonies were observed in the apiaries of Molchanovsky and Teguldetsky districts. In February, the death of some bee colonies was registered. The mass losses of bee colonies continued for a month after the first flight. The disease was characterized as a typical type A nosemosis caused by *N. apis*. The main clinical symptom was diarrhea of bees (Ostroverkhova *et al*., 2014). In all the studied samples of dead bees, both species of *Nosema* were identified, but *N. apis* was dominant. The most likely cause of the death of bee colonies in apiaries was *Nosema* disease.

Thus, in the Tomsk region, two *Nosema* species were detected – *N. apis* and *N. ceranae.* In 2016–2017, the rapid and wide spread of the more pathogenic *N. ceranae* was observed in apiaries in most studied areas. However, according to a retrospective analysis of bee colonies collected from apiaries of the Tomsk region in 2008–2011 and stored in the Bank of bee samples, *N. ceranae* have been present in some apiaries since at least 2009 (Ostroverkhova *et al*., 2018). In this regard, it is of interest to analyze the long-term dynamics of infection of honeybees with different *Nosema* species in apiaries of the Tomsk region.

Dynamics of infection of apiaries with different *Nosema* species in 2012–2017

Long-term studies of the infestation of honeybees by *Nosema* showed wave-like dynamics of infection of apiaries in the Tomsk region for various species of pathogens (Table, Fig. 4). If in 2013–2014, most of the studied apiaries were infected only with *N. apis* (60.0% of all infected apiaries), then in 2015–2017, *N. apis* was detected in only 21.7% of apiaries. If in 2013–2014, only 20.0% of the apiaries were infected with both species of microsporidia, then in 2015–2017, the co-infection prevailed (52.2%) in the apiaries. The number of apiaries infected with only *N. ceranae* was about the same in 2013–2015 and 2016–2017 and was about 20%.

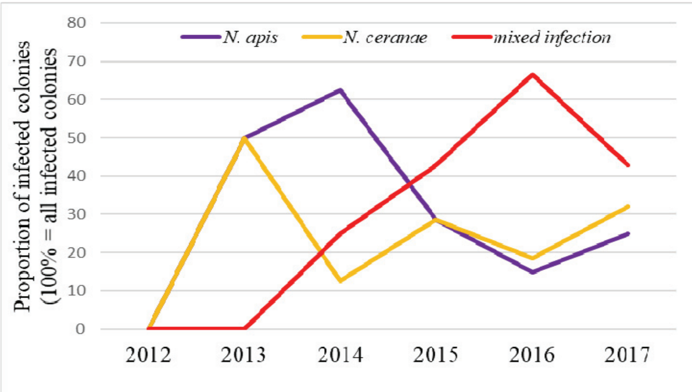


Fig. 4. Long-term dynamics of infestation of apiaries with different *Nosema* species in 2012–2017. The prevalence of samples positive for only *N. apis* (in blue), only *N. ceranae* (yellow), and both species (red) are shown at each time point. *Nosema* differentiation was performed by PCR.

Thus, during the period studied, the infection of honeybees with *Nosema* microsporidia increased; bee colonies infected with both species are predominant. Despite the wide distribution of *N. ceranae* in apiaries of the Tomsk region, replacement of the species *N. apis* by another parasite *N. ceranae* is not observed. Our results are consistent with data of long-term

Table – Distribution of two species of *Nosema* microsporidia in the apiaries of the Tomsk region in 2012–2017.

Sampling year	Number of apiaries analyzed	Infection of apiaries with microsporidia
2012	15	Microsporidia *Nosema* are not registered in any of the investigated apiary.
2013	40	Only two apiaries were infected with *Nosema*. In one apiary, the pathogen *N. apis* was detected, in the other apiary – *N. ceranae*. This was the first registered case of infection of honey bees with the more aggressive pathogen *N. ceranae* in an apiary of the Tomsk region by PCR (Ostroverkhova *et al*., 2014).
2014	44	Of the 8 infected apiaries, only *N. apis* was detected in 5 apiaries (62.5%), only *N. ceranae* was identified in one apiary (12.5%), and both *Nosema* species were registered in two apiaries (25.0%).
2015	32	Of the 14 infected apiaries, only *N. apis* was detected in 4 apiaries (28.6%), only *N. ceranae* was identified in 4 apiaries (28.6%), and both *Nosema* species were registered in 6 apiaries (42.8%).
2016	39	Of the 27 infected apiaries, only *N. apis* was detected in 4 apiaries (14.8%), only *N. ceranae* was identified in 5 apiaries (18.5%), and both *Nosema* species were registered in 18 apiaries (66.7%).
2017	37	Of the 28 infected apiaries, only *N. apis* was detected in 7 apiaries (25.0%), only *N. ceranae* was identified in 9 apiaries (32.1%), and both *Nosema* species were registered in 12 apiaries (42.9%).

studies on the prevalence of the *Nosema* parasites in the honeybee population of Northeast Germany: starting from a very low level, the prevalence of *N. ceranae* infections significantly increased during the last 12 years, but there is no general replacement of *N. apis* (Gisder *et al*., 2017).

At the same time, reports on interactions between of the species *Nosema* in honeybees from different territories of Europe are contradictory, and different prevalence of parasites in various climatic conditions have been described (Klee *et al*., 2007; Chen *et al*., 2008; Williams *et al*., 2008; Invernizzi *et al*., 2009; Chen & Huang, 2010; Stevanovic *et al*., 2011; Yoshiyama & Kimura, 2011; Copley *et al*., 2012). In countries with hot summers and moderate winters, *N. ceranae* is predominant and nearly replaced *N. apis* over the past decade in Spain and Italy (Higes *et al*., 2006, 2008; Klee *et al*., 2007; Martín-Hernández *et al*., 2007). On the contrary, in countries with rather cold and long winters such as Sweden and Germany, according to 12-year research, *N. apis* is prevailing species (Gisder *et al*., 2010, 2017).

It is assumed that the cold climate is one of the limiting factors of *N. ceranae* distribution, as *N. ceranae* spores are capable of surviving high temperatures (60°C) and desiccation, but they are intolerant of cold (4°C) (Fries, 2010; Gisder *et al*., 2010). In warmer climates, *N. ceranae* is more competitive than *N. apis* (Gisder *et al*., 2010).

In the Tomsk region, *N. ceranae* is widespread in apiaries, but mixed infection with *N. apis* dominates in bee colonies. We found *N. ceranae* infected bee colonies in cold climate with long winters and humid summers, and this parasite is not associated with colony depopulation or honeybee collapse. It should be noted that in Siberia, the honeybee *A. mellifera* is an artificial population whose wintering is controlled by people. Perhaps this fact, combined with climate change, may contributed to the spread of *N. ceranae* in Siberia.

It should be noted that most of the studies on the seasonality of *N. ceranae* or replacement of the traditional parasite *N. apis* by *N. ceranae* in honeybees are short-termed studies, usually not more than two years, with a limited number of samples (Gisder *et al*., 2017). Therefore, the long-term studies will contribute to understanding the interaction between the two species of microsporidia and the role of various factors, primarily climate, in the spreading of these important honeybee parasites.

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AUTHOR CONTRIBUTIONS

All authors have contributed equally to the work. The authors declare that they have no potential conflict of interest in relation to the study in this paper.

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